MACROMOLECULAR SWITCHES FOR BILAYER MEMBRANES

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ABSTRACT

Controlled polyelectrolyte adsorption provides a powerful tool for use in the design of thin molecular films. The adsorption of polyelectrolyte chains would be expected to cause large changes in the delicate balance of forces that determines the structural and functional properties of organized assemblies, including monolayer, bilayer and multilayer films. But in addition, polyelectrolyte adsorption is exceedingly sensitive to environment, e.g., to pH, temperature and ionic strength. Controlled adsorption can therefore serve as a molecular switching mechanism, rendering film properties sensitive to selected chemical and physical stimuli. We have used this approach to effect molecular switching in mixtures of poly(2-ethylacrylic acid) with bilayer vesicles of natural or synthetic phosphatidylcholines. In particular, we have prepared phosphatidylcholine vesicles that release their contents rapidly and quantitatively in response to changes in pH, temperature or glucose concentration. The design and preparation of responsive bilayer membranes, and the kinetics and mechanisms of the associated molecular switching processes, are discussed herein.

The natural bilayer membranes that surround cells and subcellular organelles are remarkably lively and interactive structures. Their most rudimentary function — that of defining and partitioning the cellular volume — is only one of many tasks performed by these membranes. In addition, they recognize other cells, they bind drugs and hormones, they control mass transport into and out of the cell, and they are involved in an intimate way in cell division, in energy transduction, in chemical synthesis and in information flow. Such membranes cannot be static; their properties must be variable and subject to control by an array of distinct chemical and physical signals. The study of biomembrane signalling processes is presently an area of enormous research activity (1).

The process of synaptic transmission provides a particularly striking example of the ways in which membrane response can be used to control complex biological function. Figure 1 shows a junction, or synapse, formed by a neuron and the niuscle cell stimulated by it. Comniunication between these cells is accomplished by the release of acetylcholine from the neuron, diffusion of acetylcholine across the synaptic cleft, and binding of the neurotransmitter to the surface of the postsynaptic membrane. But what triggers the release of acetylcholine? While this is still a subject of some controversy, the conventional view (2) is that acetylcholine is packaged in the neuron in membrane-bounded synaptic vesicles, and that neurotransmitter release results from fusion of the vesicular and axonal membranes. The mechanism of membrane fusion, and the precise nature of the signal that drives it, are not fully understood, but it is clear that membrane depolarization and elevated concentrations of calcium ion are required for neurotransmitter release. Thus the enormously complex process of locomotion is controlled at

the level of the niuscle cell by a conceptually simple process of signal-sensitive release of a chemical substance from a vesicular storage site.

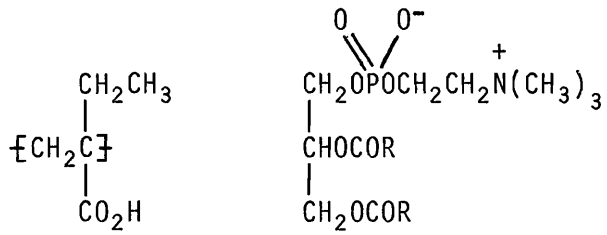
We have begun to develop synthetic vesicle systems that will release their contents in similar fashion, i.e., in response to well-defined chemical and physical signals (3-5). Our approach has been to use synthetic polyelectrolytes to control the self-assembly of membrane-forming surfactants, and then to exploit the conformational transitions of those polyelectrolytes to "switch" the structural and functional properties of the surfactant membrane.

The advantages of polyelectrolytes in this role are two-fold. First, the cooperativity characteristic of macromolecular processes allows us to niake very sharply defined ("on-off") changes in the critical properties of the membrane. Second, we can design into a single macromolecule several different functions; e.g., a single chain might carry distinct functional groups designed to: i). anchor the chain on the membrane surface, ii). receive the signal and iii). reorganize the membrane structure. Molecular switches 1acking macroniolecular size cannot accomniodate such multiplicity of function.

We describe in the present paper our work on the development of macromolecular switches that can be used to create phospholipid vesicle membranes that are sensitive to pH, temperature or glucose concentration. Very substantial contributions to the development of molecular switches for bilayer membranes have been made independently by Shinkai, Kajiyama, Kunitake and Okahata (6), but because their switching mechanisms are quite different from our own, we will not discuss this work here. The reader is encouraged to consult ref. 6 for leading references to these very interesting papers.

The Poly(2-Ethylacrylic Acid)-Phosphatidylcholine System. Most of our work to date has been concerned with the interactions of poly(acrylic acid)

derivatives with vesicle membranes prepared from phosphatidylcholines. In particular, poly(2-ethylacrylic acid) (PEAA, 1) is a hydrophobic poly(carboxylic acid) known to undergo a conformational transition from an expanded coil at high pH to a globular structure in acidic aqueous solutions (7-9). That this conformational transition occurs near neutral pH makes PEAA a candidate for use in molecular switching processes in physiologic fluids, and perhaps for pH-controlled drug delivery to body compartments more acidic than the nornial circulatory system.



1

2 a: R = $C_{15}H_{31}$
b: R = mixed hydrocarbon chains
c: R = $C_{13}H_{27}$
d: R = $C_{11}H_{23}$

The phosphatidylcholines (2) are convenient as a source of pure, structurally variable surfactants capable of forming stable bilayer membranes in aqueous suspensions. In addition, of course, they are the predominant lipids in the extracellular monolayer of mammalian cell membranes, and so are the natural choice for fabrication of vesicular systems for delivery of drugs and other biological materials (10). Phosphatidylcholines, like other double-chain surfactants, can be forniulated into single- or multiple-walled vesicles through a variety of well-established procedures (11,12).

Figure 2 presents the working hypothesis that has motivated much of our investigation of the PEAA-phosphatidylcholine systeni. The suggestion is that

protonation of the acidic sites on PEAA drives the polyelectrolyte chain to the membrane surface, and that collapse of the chain triggers reorganization of the surfactant from vesicular to micellar form. Figure 2 is not meant to imply that each micelle contains a single polymer chain or that the chain is buried in the micellar core. The following sections examine the validity of our working hypothesis and its exploitation in the design of phosphatidylcholine membranes sensitive to pH, temperature or glucose concentration.

pH-Sensitive Phosphatidylcholine Membranes. Figure 2 makes three readily testable predictions concerning the changes that should accompany acidification of phosphatidylcholine suspensions in aqueous solutions of PEAA. First, structural reorganization of the surfactant from vesicular form into mixed micelles should cause a large decrease in aggregate size. Multilamellar phosphatidylcholine vesicles are typically several hundred nanometers in diameter, whereas micelles should be of sizes comparable to twice the surfactant chain length (ca. 5 nm). Second, the hydrocarbon chain order characteristic of multilamellar vesicles should be lost, since chain packing in the micellar aggregate should be disrupted by polymer-lipid mixing and by the highly curved aggregate geometry. Finally, Figure 2 suggests that a depression of pH should lead to quantitative release of any vesicle-entrapped, water-soluble substances.

Figure 3 shows that our expectations regarding aggregate size and hydrocarbon chain packing are fulfilled. The Figure shows a plot of the optical density of a suspension of dipalmitoylphosphatidylcholine (DPPC, 2a) in an aqueous solution of PEAA, over the pH range 5.9 to 7.6. The optical density drops sharply between pH 6.7 and pH 6.4 as the suspension is acidified, and suggests that the surfactant is indeed reorganized into smaller aggregates at

low pH. The structural transition is remarkably abrupt, and supports our assertion that macromolecular species may be uniquely useful in effecting "on-off" switching in bilayer films. That the change in optical density is in fact a result of a change in aggregate size is supported by measurements of particle diffusion rates via a quasielastic light scattering technique (13).

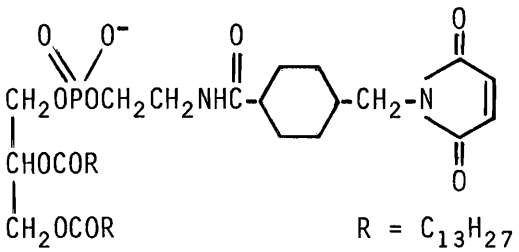
Figure 3 also shows the pH-dependence of the width ($\Delta T_{1/2}$) of the thermally induced phase transition in the DPPC bilayer. Pure DPPC is pH-insensitive over this range, but the PEAA-modified sample melts sharply only at high pH. The transition half-width increases rapidly between pH 6.7 and pH 6.4, and in more acidic solutions the phase transition is virtually absent. The absence of a phase transition is consistent with micellar aggregation.

Figure 4 shows the results of the functional test of the PEAA-phosphatidylcholine system. The Figure shows the efflux of a dye (carboxyfluoroescein) from unilamellar vesicles of egg yolk phosphatidylcholine ($\underset{\sim}{2}\underset{\sim}{b}$) suspended in aqueous PEAA. The escape of the dye is slow at pH 7.4, but the vesicle contents are delivered rapidly and quantitatively to the extravesicular space upon acidification to pH 6.5. We have since demonstrated the pH-triggered release of other substances from phosphatidylcholine vesicles by this method, and the technique should be completely general in its application to the controlled release of water-soluble compounds.

Surface-Anchoring of Poly(2-Ethylacrylic Acid). The bilayer switching processes described in the preceding sections rely on adsorption-desorption equilibria to effect structural reorganization of the surfactant aggregate. Such processes are of course impractical in controlled drug delivery. Instead, the polyelectrolyte chain must be bound irreversibly to the membrane surface, so that the systemic polyelectrolyte concentration is effectively

zero. Under conditions of high pH, such a vesicle carries a surface coat of expanded polyelectrolyte chains. Acidification will then serve to collapse the surface layer with rupture of the vesicular membrane and release of vesicle contents (Figure 5).

A system of the kind represented schematically in Figure 5 has recently been prepared by Mizuo Maeda of this laboratory (14). The method involves the coupling of thiolated PEAA to egg phosphatidylcholine vesicles containing a few percent of the maleimido phospholipid 3. Michael addition of the



polymer-bound thiol groups to the maleimide functions of the surface results in immobilization of ca. 60 μg of PEAA per mg of lipid. Following fractionation of the sample on a size exclusion column to separate vesicles from free PEAA, acidification of the vesicle suspension causes rapid release of vesicle contents. These results demonstrate that it is indeed possible to anchor sufficient PEAA to effect useful changes in membrane structure and function. We believe that anchored systems of this kind show particular promise in controlled release technologies.

Temperature-Dependence of Membrane Reorganization. The polyelectrolyte-induced rupture of egg yolk phosphatidylcholine vesicles following acidification at room temperature is rapid, as implied by the prompt release of entrapped carboxyfluorescein shown in Figure 4. In general, however, the rate

of membrane reorganization is a strong function of the phase diagram of the surfactant aggregate. For a given surfactant, then, the rate of reorganization is highly dependent on temperature. As an example, the loss of optical density associated with the PEAA-induced rupture of vesicles of dimyristoylphosphatidylcholine (DMPC, 2c) is characterized by a half-time of several hours at 19°C, as compared to a half-time of 20-25 seconds at 24°C, the main phase transition temperature (5).

This observation allows one to formulate vesicles that will release their contents in response to small changes in temperature. It is necessary only to work below the main phase transition temperature of the lipid bilayer, and to adjust the pH to a value more acidic than the "critical" pH for membrane reorganization. The result is a kinetically "stable" membrane that will rupture on warming through the phase transition, with rapid and quantitative release of vesicle contents.

Figure 6 shows the results of an evaluation of this idea. The Figure shows the temperature dependence of the optical density of a suspension of DPPC in a mildly acidic (pH 6.5) solution of PEAA. The optical density remains high as the suspension is warmed from room temperature to approximately 41°C, whereupon melting of the bilayer results in rapid reorganization of the surfactant into micellar aggregates. Although we have not determined the temperature dependence of the permeability of this system, it is virtually certain that membrane reorganization would result in quantitative release of vesicle contents. One might envision the use of such systems in the controlled delivery of diagnostic or therapeutic agents to regions of local hyperthermia, since the rates and magnitudes of the permeability changes

achieved in these systems would be expected to be much greater than those that are intrinsic to the bilayer phase transition.

Glucose-Dependent Disruption of Phospholipid Vesicle Membranes (5). Professor A S. Hoffman suggested to us a short time ago that we might combine the chemistry just described with enzymatic generation of H^+, because in doing so we should produce membranes that would be sensitive to small concentrations of the organic compounds that serve as substrates for those enzymatic reactions. While this idea is quite general, Professor Hoffman suggested specifically that we might begin with enzymatic oxidation of glucose to gluconic acid. Such a glucose-sensitive membrane system might find application in self-regulated insulin delivery or in monitoring of glucose concentrations in physiologic fluids.

Figure 7 shows the results of an experiment in which dilauroylphosphatidylcholine (DLPC, 2d) was suspended in an unbuffered solution of PEAA and glucose oxidase at pH 7.4. After several minutes during which the turbidity of the suspension remained stable, glucose was added at a concentration of 1.3 mg/mL (17). The optical density of the suspension then fell rapidly, and reached a value less than 10% of the original after approximately 30 min. Control experiments in which glucose was added to polymer-free suspensions revealed no loss of turbidity on similar timescales.

We have not determined the permeability changes associated with the membrane reorganization illustrated in Figure 7, but as before, we assert that the barrier properties of the membrane must be lost. This technique thus allows us to render membrane properties sensitive to glucose, and by inference, to many other substrates that may be converted to acidic products by oxidative or hydrolytic processes. The technical problems to be overcome in practical

applications of this concept are substantial, but the fundamental problem of control of membrane structure has been overcome.

CONCLUSIONS

Controlled adsorption of poly(2-ethylacrylic acid) can be used to render phosphatidylcholine vesicles sensitive to pH, to temperature or to glucose concentration. Poly(2-ethylacrylic acid) can also be anchored irreversibly on vesicle surfaces, in amounts sufficient to effect rapid molecular switching. The mechanism of membrane reorganization has been identified as a process akin to a vesicle-to-micelle transition. Applications of switchable vesicle systems in biology and medicine are readily imagined.

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FIGURE CAPTIONS

Figure 1. Schematic illustration of synaptic transmission at the neuromuscular junction. Membrane depolarization coupled with an influx of Ca^{2+} (top) stimulates fusion of the axonal membrane and the membranes of some number of synaptic vesicles (bottom). Acetylcholine is released into the synaptic cleft and binds to receptors on the surface of the postsynaptic membrane. Adapted from ref. 2.

Figure 2. Working hypothesis concerning mechanism of pH-dependent structural reorganization in mixtures of poly(2-ethylacrylic acid) and phosphatidylcholines. From D. Y. Takigawa and D.A. Tirrell, Makromol. Chem. Rapid Comniun. 6 (1985), 653.

Figure 3. pH-Dependences of optical density (O.D.) and calorimetric phase transition width ($\Delta T_{1/2}$) in suspensions of DPPC in aqueous PEAA solutions. The PEAA used in these experiments carried approximately 1.5 x 10^{-3} mol-% pyrene as a fluorescent label. K.A. Borden, K.M. Eum, K H. Langley and D.A. Tirrell, Macromolecules 20 (1987), in press.

Figure 4. Efflux of carboxyfluorescein from sonicated egg yolk phosphatidylcholine vesicles suspended in 50 mM Tris-HCl, 100 mM NaCl at indicated pH. From ref. 4.

Figure 5. Schematic illustration of vesicle disruption by surface-bound polyelectrolyte. The darkened rectangles represent hydrophobic anchoring groups attached in small amounts to the polyelectrolyte chain.

Figure 6. Optical density (400 nm) of a multilamellar suspension of DPPC in aqueous PEAA, pH 6.5, as a function of temperature.

Figure 7. Optical density of a multilamellar suspension of DLPC in an aqueous solution of PEAA and glucose oxidase, prior and subsequent to addition of glucose. From ref. 5.

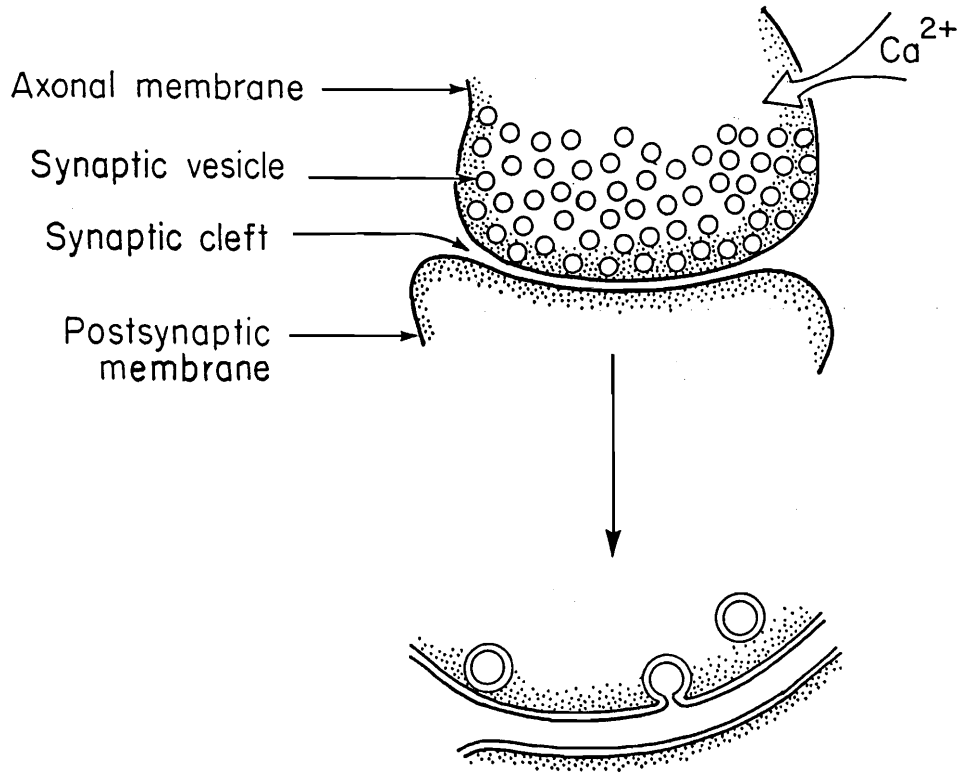
Axonal membrane
Synaptic vesicle
Synaptic cleft
Postsynaptic membrane
Ca2+

Figure 1

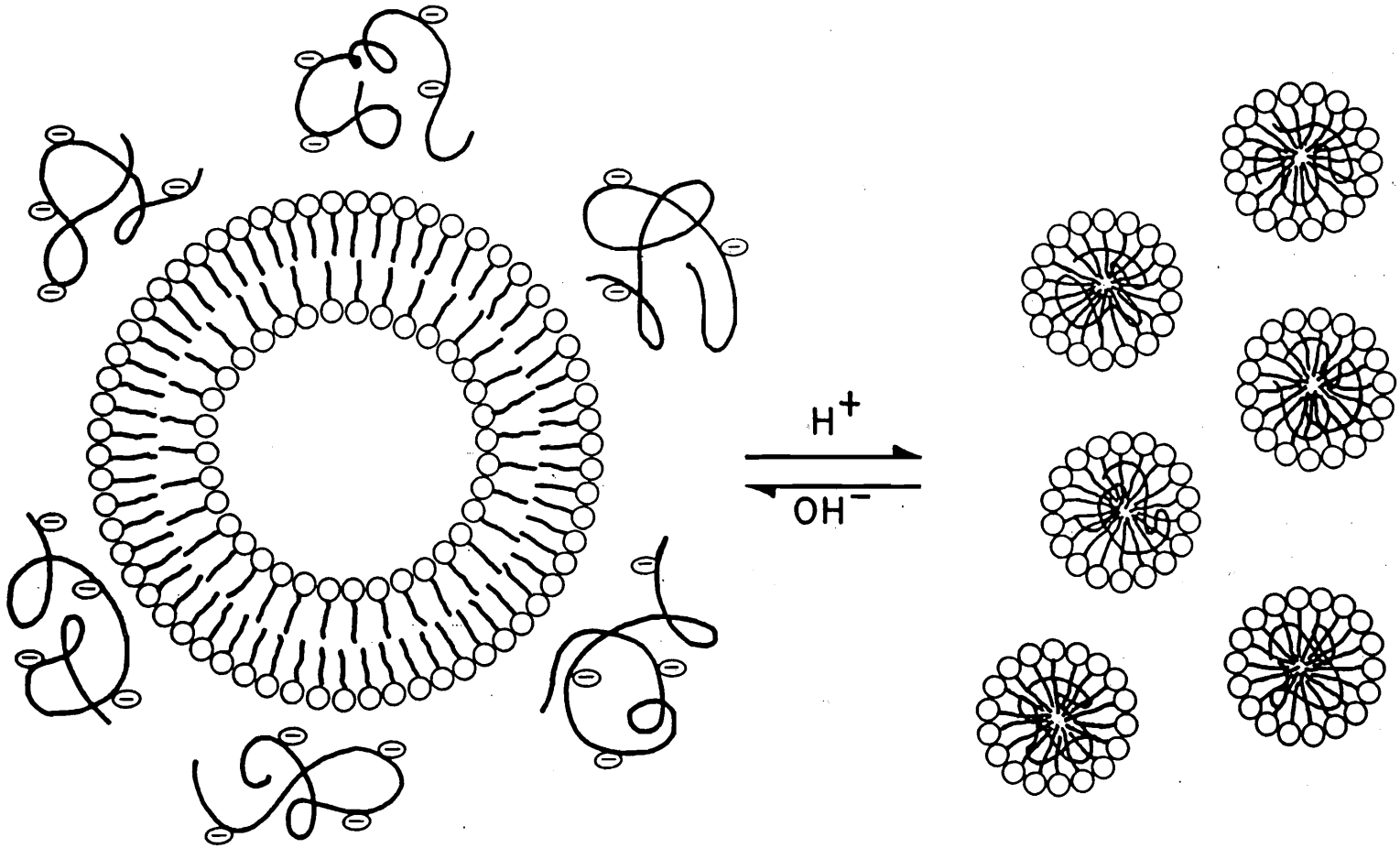

Figure 2

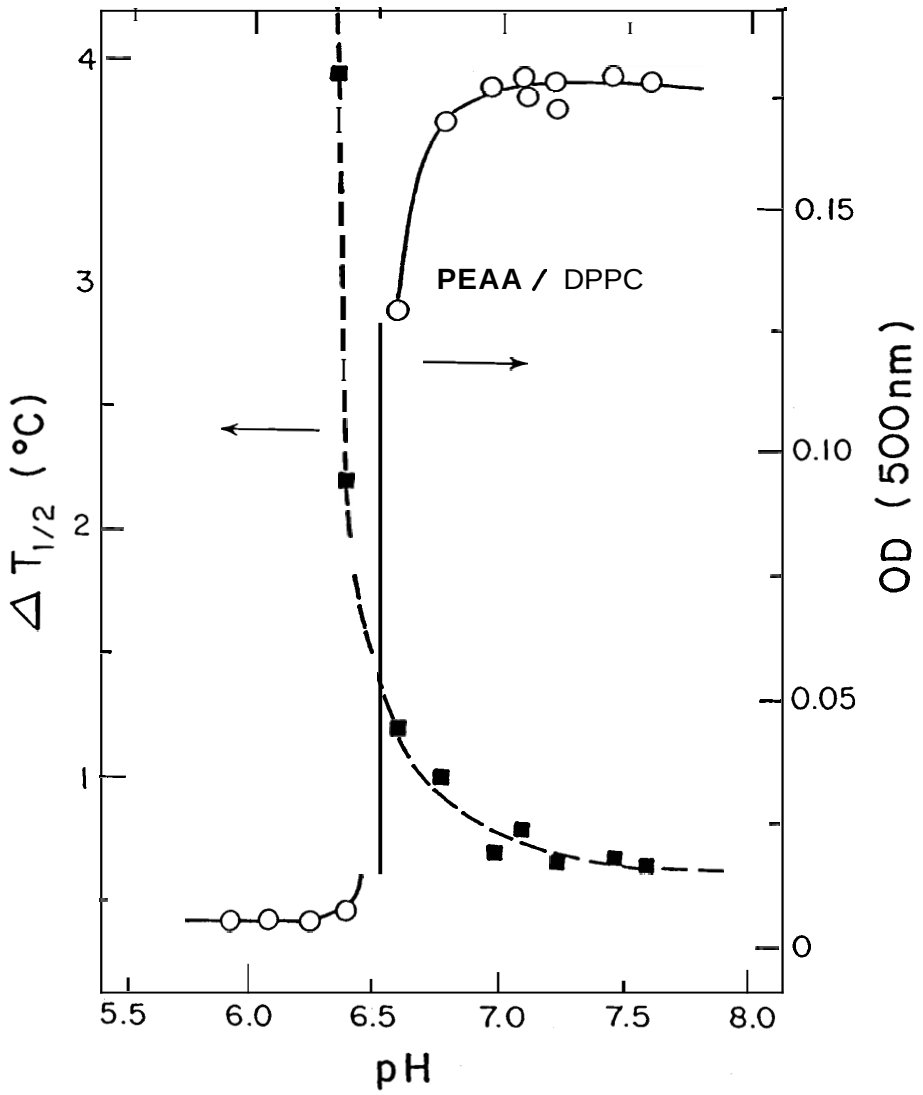

Figure 3

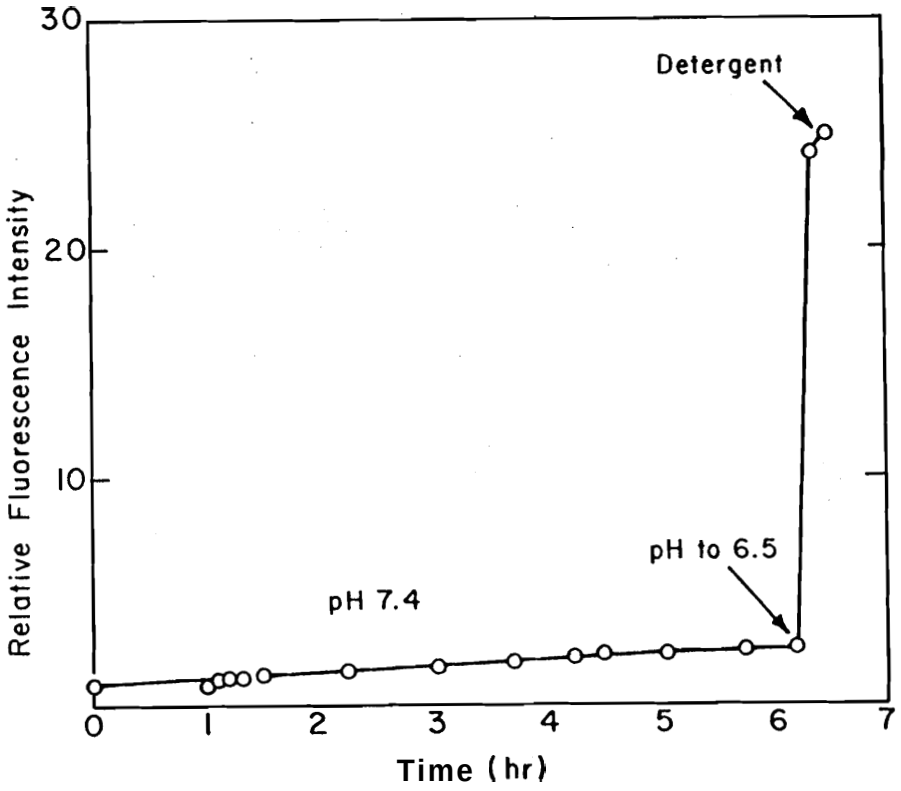
30
20
10
Relative Fluorescence Intensity
Detergent
pH to 6.5
pH 7.4
0
1
2
3
4
5
6
7
Time (hr)

Figure 4

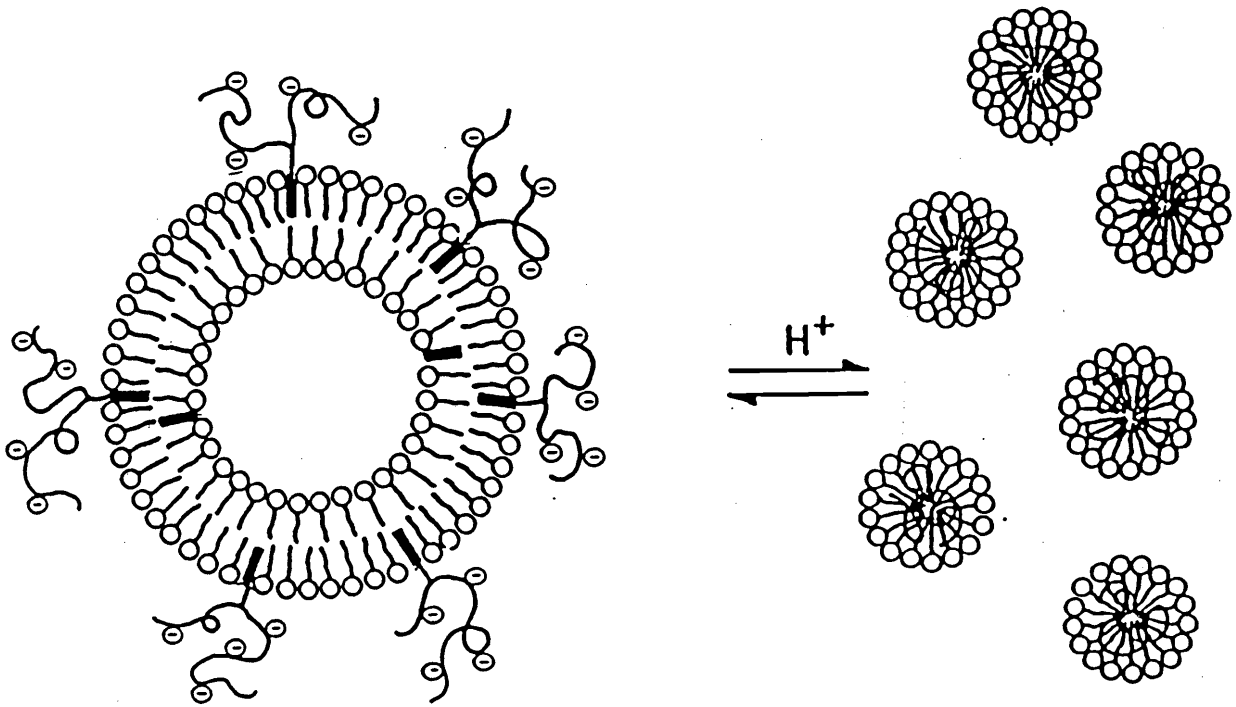

Figure 5

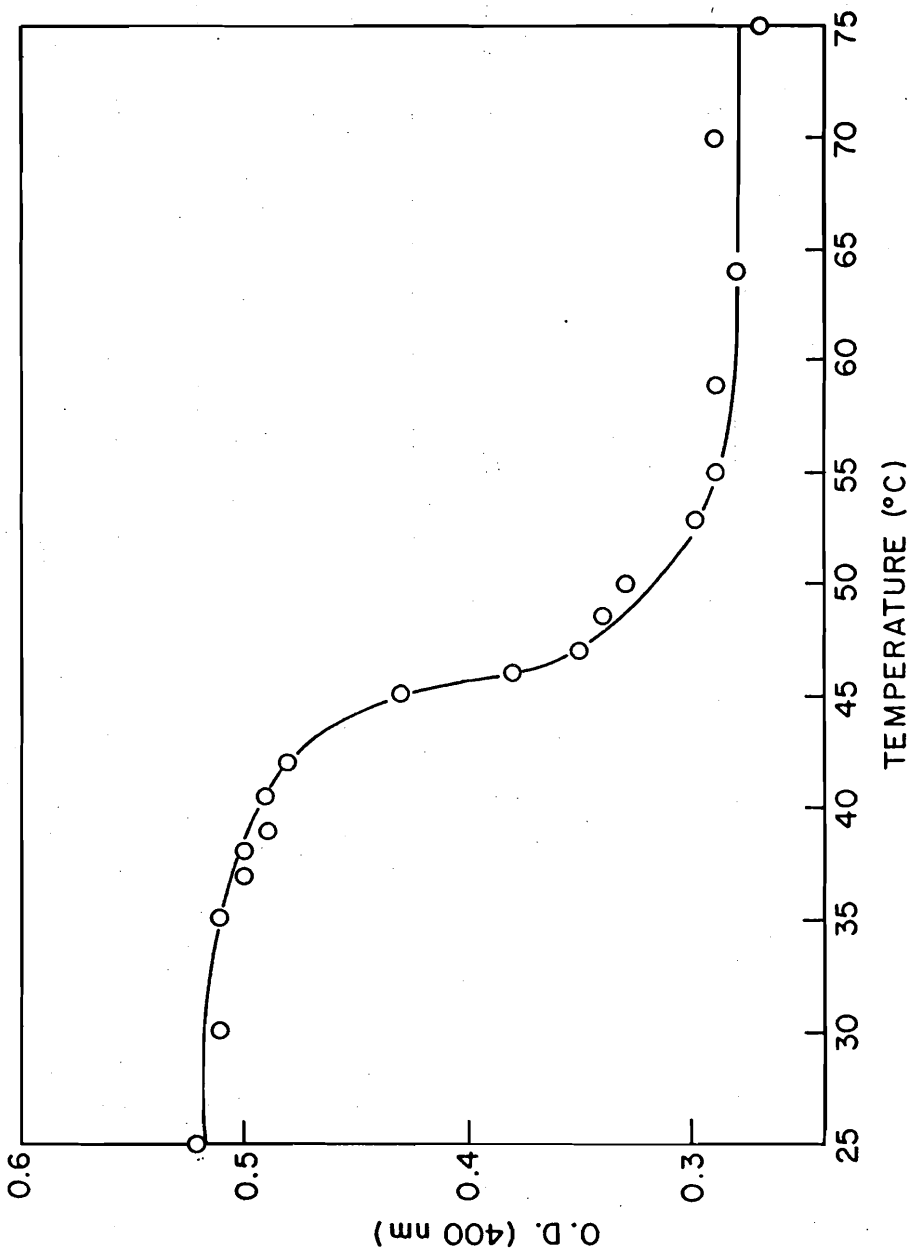

Figure 6

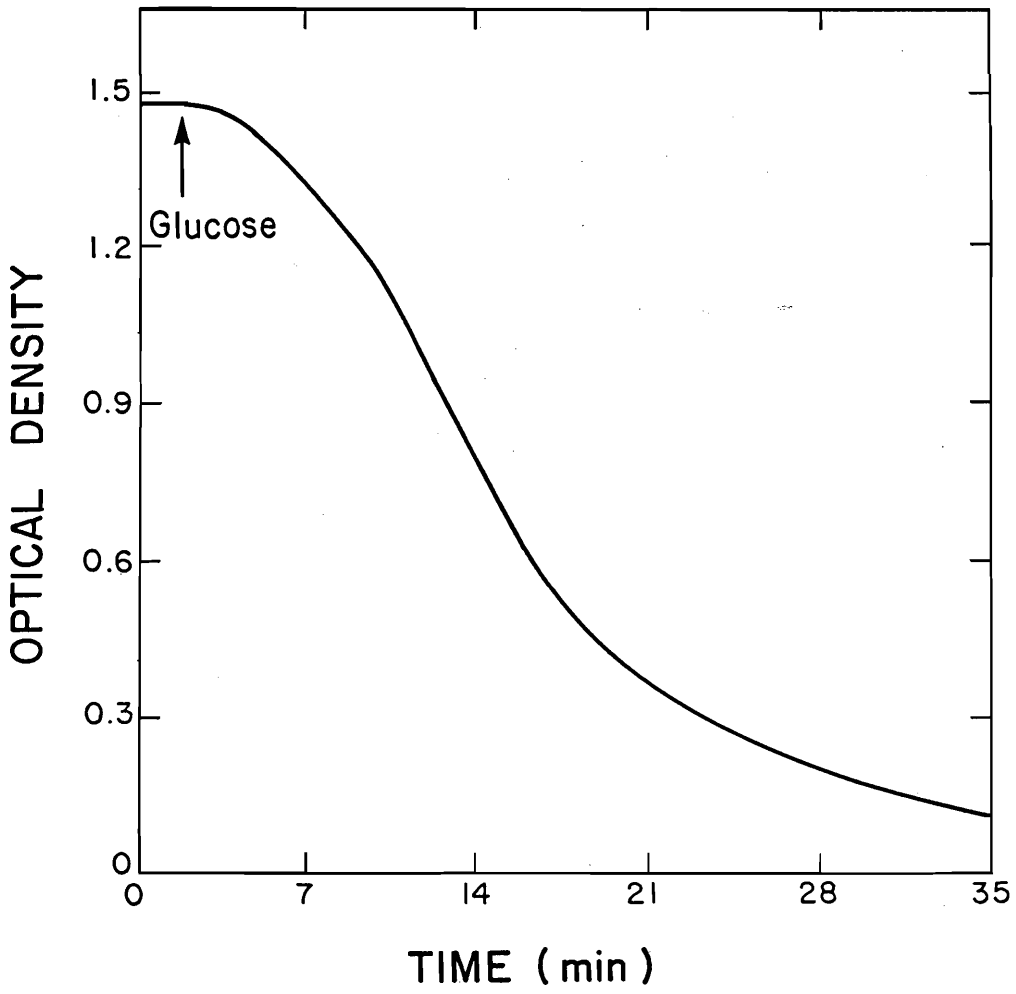

Figure 7